

PYRIMIDINE TARGETING HAIRPIN TRIPLEX-FORMING OLIGONUCLEOTIDES

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Abstract

Hairpin triplex-forming oligonucleotides that target pyrimidine nucleic acids are disclosed. The oligonucleotides of the invention are characterized by having a duplex-forming region, a triplex-forming region, and a linker region wherein one of the internucleoside linkages between the duplex-forming and triplex-forming region is a 5'-5' or 3'-3' linkage. The duplex-forming region is comprised of purine nucleosides and has a sequence substantially complementary to a pyridine region of a target nucleic acid. The triplex-forming region is comprised of pyrimidine nucleosides and is substantially complementary in the Watson-Crick sense to the duplex-forming region. The linker region is comprised of nucleotides or other moieties that link the duplex- and triplex-forming regions. In the absence of target, the hairpin triplex-forming oligonucleotide folds back upon itself, the duplex- and triplex-forming regions running in parallel. In the presence of target nucleic acids, the duplex-forming region binds to the target by Watson-Crick bonding, and the triplex-forming region by Hoogsteen bonding, forming a triplex. The disclosed oligonucleotides display increased stability and are useful for modulating gene expression.

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We claim: 1. A hairpin triplex-forming oligonucleotide comprising a duplex-forming region, a triplex-forming region, and a linker region, wherein the duplex-forming region is complementary to a pyrimidine region of a target nucleic acid and is able to hybridize to that region and thereby form a duplex, and wherein the triplex-forming region is complementary to the duplex-forming region and is able to hybridize to both the duplex-forming region, thereby-forming a hairpin duplex, as well as to the duplex formed between the duplex-forming region and the pyrimidine region of the target nucleic acid, thereby-forming a triplex, and wherein the duplex-forming region and triplex-forming region have opposite polarity and are connected to each other by the linker region.

2. An oligonucleotide according to claim 1 wherein the linker is a nonnucleotide linker.

3. An oligonucleotide according to claim 2 wherein the linker is hexaethylene glycol.
4. An oligonucleotide according to claim 1 wherein the linker region comprises from 3 to about 10 nucleotides in length and one of the linkages between the duplex-forming region and the triplex-forming region is a 3'-3' or 5'-5' internucleoside linkage.

5. An oligonucleotide according to claim 4 wherein the linker is about 5 nucleotides in length.

6. An oligonucleotide according to claim 4 wherein the duplex-forming region is from about 8 to about 50 nucleotides in length.

7. An oligonucleotide according to claim 5 wherein the duplex-forming region is from about 25 to 35 nucleotides in length.

8. An oligonucleotide having sequence 5'-AGGAAGAAAGAAAAA-3'- 3'-GGAGGTTTTTCTTTCTTCCT-5' (SEQ ID NO 3).

9. An oligonucleotide having sequence 3'-AAAAAAGAAAGAAGGA-5'- 5'-GGAGGTCCTTCTTTCTTTTTT-3' (SEQ ID NO 4).

10. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 1.

11. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 2.

12. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 3.

13. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 4.

14. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 5.

15. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 6.

16. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 7.

17. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 8.

18. A method of modulating the expression of a nucleic acid having a homopyridine sequence, the method comprising contacting the nucleic acid with a hairpin triplex-forming oligonucleotide according to Claim 9.

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PYRIMIDINE TARGETING
HAIRPIN TRIPLEX-FORMING OLIGONUCLEOTIDES
BACKGROUND OF THE INVENTION

Field Of The Invention

The invention relates to synthetic oligonucleotides. More particularly, the invention relates to synthetic oligonucleotides that are useful in gene expression modulation.

Summary Of The Related Art

Since Zamecnik and Stephenson, *Proc. Natl. Acad. Sci. USA* 75, 280284 (1978) first demonstrated virus replication inhibition by synthetic oligonucleotides, great interest has been generated in oligonucleotides as therapeutic agents. In recent years the development of oligonucleotides as therapeutic agents and as agents of gene expression modulation has gained great momentum. The greatest development has been in the use of so-called antisense oligonucleotides which form Watson-Crick duplexes with target mRNAs. Agrawal, *Trends in Biotechnology* 10, 152-158 (1992) extensively reviews the development of antisense oligonucleotides as antiviral agents.

Also important, but somewhat less advanced, is the so-called antigene oligonucleotide approach in which oligonucleotides form triplex with target DNA duplexes through Hoogsteen base pairing. Thoung and Helene, *Angew. Chem. Int. Ed. Engl.* 32: 666-690 (1993) have recently reviewed developments in this latter approach. See also Uhlmann and Peyman, *Chem. Rev.* 90:543-584 (1990) and Chang and Pettitt, *Prog. Biophys. Mol. Biol.* 58:225-257 (1992).

Chem. Int. Ed. Engl. 32: 666-690 (1993) have recently reviewed developments in this latter approach. See also Uhlmann and Peyman, *Chem. Rev.* 90:543-584 (1990) and Chang and Pettitt, *Prog. Biophys. Mol. Biol.* 58:225-257 (1992).

Triplex formation has been observed between DNA and various types of oligonucleotides. Cooney et al., *Science* 241:456-459 (1988) teaches triplex formation between DNA and an oligodeoxynucleotide phosphodiester. Latimer et al., *Nucleic Acids Res.* 22, 1549-1561 (1989) discloses triplex formation involving oligodeoxynucleotide phosphorothioates. Kibler-Herzog et al., *Nucleic Acids Res.* 18, 3545-3555 (1990) discloses triplex formation involving short oligodeoxynucleotide methylphosphonates. Various base modifications that enhance triplex formation are also known, including C5-methylation of cytosine (Xodo et al., *J. Molec. Biol.* 19, 5625-5631 (1991)), use of the bicyclic cytosine analog, MODA (Young et al., *Proc. Natl. Acad. Sci. USA* 88, 10023-10100 (1991)), and use of a synthetic α -anomeric nucleotide (Praseuth et al., *Proc. Natl. Acad. Sci. USA* 85, 1349-1353 (1988)). Giovannangeli et al., *J. Am. Chem. Soc.* 113, 7775-7777 (1991) teaches that attachment of an acridine intercalator onto the 3'-end of a capped oligonucleotide also enhances triplex stability. Oligonucleotide-mediated triplex formation can cause inhibition of transcription, at least in vitro (see Cooney et al. and Young et al., supra).

In recent years, several groups reported circular or foldback triplex formation at homopurine single stranded sites using linear (Kandimalla and Agrawal, *Gene* 149, 115-121 (1994); Xodo et al., *Nucleic Acids Res.* 18, 3557-3564 (1990); D'Souza et al., *Biomol. Struct. Dyn.* 10, 141-152 (1992); Noll et al., *Nucleosides Nucleotides* 13, 997-1005 (1994)), circular (Kool, J.

Am. Chem. Soc. 113, 6265-6266 (1991); Prakash and Kool, *J. Am. Chem. Soc.*

114, 3523-3528 (1992); and D'Souza and Kool, *Bioorg. Med. Chem. Lett.* 4, 965-970 (1994)), and ligand conjugated linear (Giovannangeli et al., *J. Am.*

Chem. Soc. 113, 7775-7776 (1992); Giovannangeli, *Proc. Natl. Acad. Sci. USA* 90, 10013-10017 (1993); and Gryaznov and Lloyd, *Nucleic Acids Res.* 21, 5909-5915 (1993)) homopyrimidine oligonucleotides, which may be useful as agents for gene expression control at translation level by targeting mRNA. The linear oligonucleotides that form foldback triplexes with homopyrimidine strand were shown to disrupt guanine quadruplex structures if the target site contains such quadruplex-forming base sequences. Kandimalla and Agrawal, *Nucleic Acids Res.* 23, 1068-1074 (1995). These oligonucleotides were demonstrated to

bind at oligopurine sites of a double helical DNA through strand invasion mechanism by D-loop formation. Kandimalla et al. and Manning, Agrawal, S. (in press).

Requirement of acidic pH conditions and limited base recognition are impediments to full implementation of the triple helix approach. Several modified bases (Ono et al., J. Am. Chem. Soc. 113, 4032-4033 (1991); Krawczyk et al., Proc. Natl. Acad. Sci. U.S.A. 89, 3761-3764 (1992); Miller et al., Biochemistry 31, 6788-6793 (1992); Koh et al., J. Am. Chem. Soc. 114, 1470-1478 (1992); and Jetter and Hobbs, Biochemistry 32, 3249-3254 (1993) or alternate designs have been reported to overcome these problems, but with limited success. Recently, an approach for targeting single stranded pyrimidine sites using circular oligonucleotides through G-G-C and T-A-T kind of triple helix formation has been proposed in which the third strand binds antiparallel to the purine strand through reverse Hoogsteen hydrogen bonding, which is weaker than Hoogsteen hydrogen bonded triplex. Wang and Kool, J. Am.

Chem. Soc. 116, 885 (1994).

Both the antisense and antigene oligonucleotide approaches have as their goal gene expression modulation that is beneficial in understanding gene expression and in therapeutic treatment of diseases or conditions involving gene expression. Two major characteristics of oligonucleotide compounds that are well suited to meet these goals are high specificity and an ability to interfere with gene expression upon binding. Enhancement of these characteristics is always desirable. There is, therefore, a need for new oligonucleotide compounds having even greater specificity and more stable complex formation, leading to increased ability to interfere with gene expression than existing compounds.

BRIEF SUMMARY OF THE INVENTION

The invention provides novel antisense oligonucleotides for targeting pyrimidine nucleic acid strands. These antisense oligonucleotides significantly increase the number of sequences that can be targeted by triplex forming oligonucleotides and also form more stable complexes with the target nucleic acids.

Oligonucleotides according to the invention have two complementary strands, which hybridize to each other and form a duplex structure. One strand of the duplex structure contains all purines and the other contains complementary all pyrimidine bases. These two strands are attached by either 3'-3' or 5'-5' linkage so that the complementary strands form a parallelstranded hairpin-duplex. The two parallel strands are held together by Hoogsteen hydrogen bonding. We name this novel structure as parallelstranded Hoogsteen duplex. The target sequence for such an antisense oligonucleotide will have the same sequence as that of the pyrimidine strand of the parallel-stranded duplex but in an opposite orientation, i.e., it will have opposite polarity. The purine strand of the antisense oligonucleotide will bind to the target pyrimidine strand in an antiparallel fashion through Watson-Crick hydrogen bonding resulting in a triplex structure (Fig. 3).

The complex formed between the target nucleic acid and oligonucleotides according to the invention has characteristics similar to both the duplex structure of the anti sense approach and the triplex structure of the antigene approach to gene expression modulation. Because the same oligonucleotide performs both duplex and triplex-forming functions, this structure is called a hairpin triplex and oligonucleotides according to the invention, which complex with target nucleic acids to form a triplex, are called hairpin triplex-forming oligonucleotides.

Hairpin triplex-forming oligonucleotides according to the invention are useful for carrying out in vitro studies of the kinetics of duplex and triplex formation under varying parameters. They are also useful in gene expression modulation studies, both in vitro and in vivo. The use of hairpin triplex-forming oligonucleotides according to the invention

provides an easily used alternative to the laborious method of modulating gene expression by deletion mutation. Accordingly, the hairpin triplex-forming oligonucleotides are useful research tools for the in vitro determination of the role of a targeted gene in biological processes. The importance of such a use can be appreciated when one realizes that the elucidation of most known biological processes was accomplished by isolating and studying deletion mutants in vitro or in "knockout" animals..

The foregoing merely summarizes certain aspects of the present invention and is not intended, nor should it be construed, to limit the invention in any manner. All patents and other publications cited herein establish the state of the an and are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the antisense and antigene approaches to gene expression modulation.

Figure 2 shows C-GsCs and TAeT base triplets with both Watson-Crick and Hoogsteen hydrogen bonding patterns implicated in triplex formation.

Figure 3A displays an example of how the complementary duplex- and triplex-forming regions of the hairpin triplex-forming oligonucleotides of the invention are oriented, and Figure 3B shows the orientation when an intramolecular duplex is formed.

Figure 4 A and B display triple-helix-forming constructs for targeting purine and pyrimidine single strands, respectively.

Figure 5 displays the derivative of the absorbance with respect to temperature for SEQ ID NOs 1-4 and 7. Oligonucleotide numbers without and with the "+" sign indicate the derivative of melting profiles of the oligonucleotide alone due to secondary structure, and in the presence of the DNA complementary strand, respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to synthetic oligonucleotides that are useful in gene expression modulation. The invention provides synthetic oligonucleotides that have greater specificity and more stable complex formation with target nucleic acids than existing oligonucleotides.

The antisense approach and the antigene approach, which are illustrated in Figure 1, are existing oligonucleotide-mediated approaches to gene expression modulation. In the antisense approach, the target nucleic acid is a single-stranded nucleic acid and the gene expression modulating agent is a complementary oligonucleotide. The complementary oligonucleotide and the target nucleic acid form a duplex by Watson-Crick base-pairing between the complementary bases. Modulation of gene expression presumably occurs by various mechanisms, including RNase H degradation of RNA, blocking of ribosome function on RNA, or blocking of enzyme function on RNA or DNA.

In contrast, in the antigene approach, the target nucleic acid is a doublestranded nucleic acid and the gene expression modulating agent is an oligonucleotide that is capable of entering the major groove of the target duplex and forming Hoogsteen base pairs, such as those shown in Figure 2, with the purine strand of the target duplex. This interaction results in triplex formation. Gene expression modulation presumably occurs via interference with transcription.

Pyrimidine single strands can also be targeted by triple helix formation.

This approach utilizes oligonucleotides having AT and G C base paired DNA hairpins in which the two strands are aligned in a parallel fashion for complexing with the DNA and RNA and pyrimidine single strands through triple helix formation. Stable parallel stranded

(ps) base pairing of A-T and GC through Hoogsteen (Liu et al. Biochem 32, 11802-11809 (1993)) hydrogen bonding has been shown to be possible (Raghunathan et al., Biopolymers 34, 1573-158 (1994)). The present invention demonstrates that these properties can be exploited to provide oligonucleotides that target pyrimidine single-stranded nucleic acids and form more stable complexes with the target than traditional antisense oligonucleotides.

Oligonucleotides according to the invention have a duplex-forming region, a triplex-forming region, and a linker region. The duplex-forming region is complementary to the target nucleic acid and is able to hybridize with the target nucleic acid via Watson-Crick base-pairing, just as in the antisense approach. The triplex-forming region is complementary in the Hoogsteen sense to the duplex-forming region but has opposite polarity, as depicted in Fig. 3A.

That is, viewing the hairpin triplex-forming oligonucleotides of the invention as linear strands, when the sequence of one of the duplex- and triplex-forming regions is oriented 3'-5', the other is oriented 5'-3' and vice versa, as shown in Fig. 3A. As used herein, the term "polarity" means the direction of the vector pointing from the 5' to 3' direction in the primary structure of the oligonucleotide. Accordingly, when an intramolecular duplex is formed, as depicted in Fig. 3B, the duplex- and triplex-forming regions are parallel in orientation. This is also the case when an intermolecular triplex is formed with a target nucleic acid. As shown in Fig. 3B, Pym and Pun, Pyro.1 and Pu.1, . . . , Py and Pu, are all Hoogsteen pairs. In this depiction, the number of nucleosides in the triplex-forming region (m) is equal to the number in the duplex-forming region (n), but this need not be the case. The duplex-forming region can have more nucleosides than the triplex-forming region.

The duplex-forming region of a hairpin triplex-forming oligonucleotide is characterized by having a nucleotide sequence that is sufficiently complementary to a target nucleic acid sequence to hybridize to the target nucleic acid sequence under experimental or physiological conditions.

Preferably, the duplex-forming region has from about 8 to 50 nucleotides, and most preferably has from about 12 to about 35 nucleotides. The target nucleic acid can have essentially any pyrimidine nucleotide sequence. For therapeutic, medical, or experimental purposes, the duplex-forming region will preferably have a nucleotide sequence that is sufficiently complementary to hybridize under physiological or experimental conditions to the nucleotide sequence of the target nucleic acid that is involved in a particular disease state or physiological condition, or which is under investigation.

The duplex and triplex-forming regions are connected via a flexible linker or loop region, which allows the oligonucleotide to fold back upon itself and hybridize to form a Hoogsteen duplex (one in which the two strands are parallel). In the presence of a target pyrimidine nucleic acid, the duplex-forming region hybridizes in the normal Watson-Crick manner to form a duplex, and the triplex-forming region remains in Hoogsteen base-pairing in the major groove of the duplex thus formed. The result of this interaction is the formation of a highly stable triplex. Such a complex (shown in Figure 4 for two oligonucleotides according to the invention and their targets) is called a triplex.

Because oligonucleotides according to the invention are capable of forming triplexes with target nucleic acids and form a hairpin within the oligonucleotide, such oligonucleotides are hereby denoted hairpin triplex-forming oligonucleotides.

Oligonucleotides according to the invention have characteristics of both the antisense and antigene approaches. The target nucleic acid for oligonucleotides according to the invention is a single-stranded nucleic acid, as in the antisense approach, and the final complex is a triplex, as in the antigene approach.

The new constructs are shown in Fig. 4 and Table 1. *infra*. Fig. 4A depicts the strategy

reported in Kandimalla and Agrawal, supra, Xodo et al., supra, D'Souza and Kool, J. Biomol. Struct. Dyn. supra, Noll et al., supra, and Prakash and Kool, supra. Fig. 4B depicts the new strategy described herein for targeting pyrimidine (C,T,U) strands. SEQ ID NOs 3 and 4 exist in parallel double helical hairpin structure under the experimental conditions (Liu et al., supra). In the presence of a single stranded pyrimidine target sequence that is complementary to the purine strand, however, oligonucleotides SEQ ID NOs 3 and 4 should form an antiparallel duplex via Watson-Crick hydrogen bonding.

The pyrimidine strands of SEQ ID NOs 3 and 4 remain bound in the major groove of the antiparallel duplex in parallel fashion to the purine strand through Hoogsteen hydrogen bonding

As used herein, the term "complementary" means an oligonucleotide sequence that is sufficiently complementary (a) in the Watson-Crick sense to hybridize to a single stranded nucleic acid or (b) in the Hoogsteen base pairing sense to hybridize to a duplex DNA under the conditions (e.g., temperature and pH) of interest. Unless otherwise indicated, as used herein the term hairpin triplex-forming oligonucleotide refers to the oligonucleotides of the present invention.

Hairpin triplex-forming oligonucleotides of the present invention differ from previously disclosed foldback triplex-forming oligonucleotides in that in the presently disclosed hairpin triplex-forming oligonucleotides the duplex-forming region and the triplex-forming region have opposite polarity.

Furthermore, the presently disclosed oligonucleotides are designed to target pyrimidine single strands. Therefore, the duplex-forming region is comprised of all purine residues and is complementary in the Watson-Crick sense to the target pyrimidine nucleic acid, thus facilitating hybridization to the target via Watson-Crick base pairing in antiparallel fashion. The triplex-forming region is a pyrimidine sequence, to complement the duplex-forming region, but in parallel orientation.

The triplex-forming region preferably has at least about 8 nucleotides and can be of any length up to the full length of the duplex-forming region. In a preferred embodiment, the bases of the triplex-forming region include 5-bromodeoxyuridine and/or 5-methylcytosine, each of which promote Hoogsteen base pairing at or near physiological pH. The bicyclic cytosine analog MODA, a-anomeric nucleotides and/or terminal acridines, other terminal intercalators, or DNA cutting or modifying agents such as EDTA-Fell, and cc-1065, or hydrophobic or amphophilic groups such as cholesterol, cyclodextrins, or polyamines may also be present in the triplex (or duplex) forming region to promote triplex stability or target nucleic acid destruction.

The linker region of hairpin triplex-forming oligonucleotides is a flexible region that connects the duplex-forming region and the triplex-forming region.

The linker region may be an oligonucleotide having from about 1 to about 10 nucleotides. In a preferred embodiment, the linker region is an oligonucleotide having about 5 nucleotides. When the linker region is comprised of nucleotides, either the duplex-forming region or the triplex-forming region are connected to the linker via a 5'-5' or 3'-3' internucleotide linkage, or the linker region has a 3'-3' or 5'-5' internucleotide linkage.

Alternatively, the linker region can be some other flexible chemical structure, such as a substituted or unsubstituted alkyl or aryl group having about 2 to 20 carbon atoms (e.g., isopropyl, o-xylol), or ribose or 1,2-dideoxyribose chains. In a preferred embodiment, the linker region is hexaethylene glycol. At a minimum, the linker region is a single covalent bond.

In the absence of target nucleic acid, the hairpin triplex-forming oligonucleotides form

intramolecular duplexes, the triplex-forming region hybridizing to the complementary (in the Hoogsteen sense) duplex-forming region. Because the two-regions have opposite polarity, a parallel stranded duplex is formed, as opposed to the usual anti-parallel stranded duplex.

The fact that hairpin triplex-forming oligonucleotides do form both duplexes and triplexes is demonstrated by independent lines of evidence. First, under conditions that allow Hoogsteen base-pairing involving a cytosine-containing triplex-forming strand (pH 5.0, under which conditions cytosine is protonated), hairpin triplex-forming oligonucleotides form a complex with a target nucleic acid that upon thermal denaturation yields a distinct increase in A260 (each increase being indicative of a denaturation event). Fig. 5. The A260 increase occurs at about the temperatures expected for thermal denaturation of triplexes and duplexes. The higher temperature A260 increase takes place at the expected temperature for disruption of a complex formed by both Watson Crick and Hoogsteen base-pairing (Kandimalla and Agrawal, *supra*). The lower temperature A260 increase for duplexes of either antiparallel or parallel nature would be expected for disruption of a less thermodynamically stable complex involving only one of the base-pairing, Watson-Crick (anti-parallel) or Hoogsteen (parallel), base pairings. In contrast, when conditions are altered to prevent Hoogsteen base-pairing involving a cytosine-containing triplex-forming strand (pH 7.4, under which conditions cytosine is not protonated), only the lower temperature A260 increase is observed. Moreover, at physiological concentrations of polyamines (1-3.0 mM spermine), which stabilizes triplex formation, the higher A260 increase pattern is restored.

For the intact hairpin triplex-forming oligonucleotides and the various structural regions referred to herein, except where explicitly stated otherwise, structural features include, but are not limited to, ribonucleosides, 2' substituted ribonucleosides, and/or deoxyribonucleotides. The internucleotide linkages may be a natural phosphodiester linkage or an artificial linkage, such as, for example, a phosphorothioate, phosphorodithioate, phosphoramidate, alkylphosphonate, alkylphosphonothioate, sulfonate, carbamate and/or phosphotriester linkage. Moreover, such oligonucleotides encompass oligonucleotides having modifications on the bases and/or-sugar residues as well as those having nuclease-resistance conferring substituents or bulky substituents at the 3' and/or 5' end.

Certain preferred embodiments of hairpin triplex-forming oligonucleotides of the present invention are shown in Table 1.

TABLE I
Selected Foldback Triplex-Forming Oligos And An Antisense Control Oligo

SEQ
Triplex Binding Region Linker Region Duplex Binding Region
ID
NO

5' -TCCTTCTTTCTTTTT-3' 1* 3' -TCCTTCTTTCTTTTT-5' 5'-
AGGAAGAAAGAAAAA-3' 3' -GGAGG-----TTTTTCTTTCTTCCT-5' 3t 3-
AAAAAAGAAAGAAGGA-5-5-GGAGG-----TCCTTCTTTCTTTTT-3 4t 5-
TCCTTCTTTCTTTTT-----CTCTC-----TTTTTCTTTCTTCCT-3 6* 5-
TTTTTCTTTCTTCCT-----CTCTC-----TCCTTCTTTCTTTTT- 3 7*

*Targeted to 5' -AGGAAGAAAGAAAAA-3' (SEQ ID NO 5)

tTargeted to SEQ ID NO 2

Oligonucleotides SEQ ID NOs 3 and 4 showed high stability against exonucleases (snake venom or spleen phosphodiesterases), single- and doublestrand specific endonucleases (S1 nuclease and DNase I), and 10% total fetal calf serum (non-heat inactivated) due to unusual 3'-3' or 5'-5' attachment, and the formation of a stable duplex structure (Rippe and Jovine, *Biochemistry* 28:95429549 (1989), Tang and Temsamani and Agrawal, *Nucleic Acids Res.* 21:27292735 (1993)).

The new strategy described here for targeting single-stranded DNA and

RNA pyrimidine sequences by formation of triple helices using ps hairpin oligonucleotides not only significantly increases the number of sequences that can be targeted by triplex formation but also decreases the susceptibility of oligonucleotides to degradation by cellular nucleases either with or without chemical modification. The latter characteristic is significantly important in the development of therapeutic agents for in vivo uses and treatment of diseases.

Hairpin triplex-forming oligonucleotides according to the invention can be synthesized according to any of the procedures known in the art for oligonucleotide synthesis E.g., Methods in Molecular Biology, vol. 20, "Protocols for Oligonucleotides and Analogs: Synthesis and Properties" (Sudhir Agrawal, Ed., Humana Press, N.J., 1993). For example, U.S. Patent No.

5,047,524, the teachings of which are hereby incorporated by reference, teaches phosphoramidite synthesis of oligonucleotides. Alternatively, U.S. Patent No.

5,149,748, the teachings of which are hereby incorporated by reference, teaches an optimized H-phosphonate approach for oligonucleotide synthesis. For hairpin triplex-forming oligonucleotides containing non-oligonucleotide linker regions, synthesis can still be carried out according to these procedures, provided that any hydroxyl groups are first protected by an appropriate protecting group, such as a dimethoxytrityl group, that any amino groups present be protected by an appropriate protective group, such as a trifluoroacetyl group, and that one end be linked to an appropriate coupling group, such as a cyanoethyl-phosphoramidite or H-phosphonate group.

Hairpin triplex-forming oligonucleotides according to the invention are useful for a variety of purposes. First, they are useful for in vitro studies of nucleic acid triplex formation. In a hairpin triplex-forming oligonucleotide it is possible to vary many parameters, such as internucleotide linkage types, base modifications, linker length and flexibility, etc. to study the kinetics of triplex formation and disruption, which may be a biologically important process.

In addition, hairpin triplex-forming oligonucleotides can be used in place of traditional antisense oligonucleotides in tissue culture and animal models for studying gene expression. In these systems, the increased specificity and complex stability of hairpin triplex-forming oligonucleotides is beneficial. The hairpin triplex forming oligonucleotides according to the invention provide an attractive alternative to deletion mutation in the modulation of gene expression for the in vitro or in vivo determination of the role of the targeted gene in biological processes.

Finally, hairpin triplex-forming oligonucleotides are useful as therapeutic agents for diseases or physiological conditions involving expression of specific genes. The disease or condition that a particular hairpin triplex-forming oligonucleotide is useful for treating will depend upon the nucleotide sequence to which the duplex-forming region is sufficiently complementary to hybridize under physiological conditions. In many cases the nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known, and has recently been reviewed in Agrawal, Tibtech 10, 152-158 (1992). The lessons learned from antisense antivirals can be applied to duplex-forming regions. -Viral nucleic acid sequences that hybridize to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (U.S. Patent No.

4,806,463, the teachings of which are herein incorporated by reference), Herpes simplex virus (U.S. patent No. 4,689,320, the teachings of which are hereby incorporated by reference), Influenza virus (U.S. Patent No. 5,194,428, the teachings of which are hereby incorporated by reference), and Human papilloma virus (Storey et al., Nucleic Acids Res. 19, 4109-4114 (1991)). Sequences hybridizing to any of these nucleic acid sequences can be used for the duplex-forming region of hairpin triplex-forming oligonucleotides, as can nucleotide sequences complementary to nucleic acid

sequences from any other virus

Additional viruses that have known nucleic acid sequences against which hairpin triplex-forming oligonucleotides can be prepared include, but are not limited to, Foot and Mouth Disease Virus (See Robertson et al., J. Virology 54, 651 (1985); Harris et al., J. Virology 36, 659 (1980)), Yellow Fever Virus (See Rice et al., Science 229, 726 (1985)), Varicella-Zoster Virus (See Davison and Scott, J.

Gen. Virology 67, 2279 (1986), Cucumber Mosaic Virus (See Richards et al., Virology 89, 395 (1978)), Hepatitis B Virus (See Ranee and McLachlen, in Molecular Biology of Hepatitis B Virus (CRC Press, 1991)), Hepatitis C Virus (See Miller and Purcell, Proc. Natl. Acad. Sci. USA 87, 2057-2061 (1990); Proc.

Natl. Acad. Sci. USA 89, 4942-4946 (1992); J. General Virology 74, 661-668 (1993)), Human Papilloma Virus (Storey et al., supra), and Respiratory Syncytial Virus (See Collins, in The Paramyxoviruses, Chapter 4, pp. 103-162 (David W.

Kingsbury, Ed., 1991)). Alternatively, the duplex-forming region can have a nucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, *Plasmodium falciparum*, and many pathogenic bacteria. Nucleotide sequences hybridizing to nucleic acid sequences from any such pathogenic organism can form the duplex-forming region of hairpin triplex-forming oligonucleotides. Examples of pathogenic eukaryotes having known nucleic acid sequences against which hairpin triplex-forming oligonucleotides can be prepared include, but are not limited to *Trypanosoma brucei gambiense* and *Leishmania* (See Campbell et al., Nature 311, 350 (1984)), *Fasciola hepatica* (See Zurita et al., Proc. Natl. Acad. Sci. USA 84, 2340 (1987)). Antifungal hairpin triplex-forming oligonucleotides can be prepared using a duplex-forming region having a nucleotide sequence that is complementary to a nucleic acid sequence from, e.g., the chitin synthetase gene, and antibacterial hairpin triplex-forming oligonucleotides can be prepared using, e.g., the alanine racemase gene. In yet another embodiment, the duplex-forming region of hairpin triplex-forming oligonucleotides can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, FASEB J. 5, 2799-2807 (1991)), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570), and various well-known oncogenes and proto-oncogenes, such as c-myc, c-abl, and n-ras. In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes. Hypertension can be controlled by oligonucleotides that suppress the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A₂ for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in arterial wall can be inhibited by suppression of the synthesis of fatty acyl coenzyme A: cholesterol acyl transferase in arteriosclerosis; inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia. There are numerous neural disorders in which hairpin triplex-forming oligonucleotides can be used to reduce or eliminate adverse effects of the disorder. For example, suppression of the synthesis of monoamine oxidase can be used in Parkinson's disease; suppression of catechol O-methyl transferase can be used to treat depression; and suppression of indole N-methyl transferase can be used in treating schizophrenia. Suppression of selected enzymes in the arachidonic acid cascade which leads to prostaglandins and leukotrienes may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma.

Suppression of the protein expressed by the multi-drug resistance (mdr) gene, which is

responsible for development of resistance to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer. Nucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for the duplex-forming region of hairpin triplex-forming oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene or gene transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S.

Patent No. 5,107,065. Since the nucleotide sequence of the duplex-forming region can be adapted to form Watson-Crick base pairs with essentially any gene, the therapeutic spectrum of hairpin triplex-forming oligonucleotides should be very broad. Still, certain diseases are of particular interest. For example, a variety of viral diseases may be treated by hairpin triplex-forming oligonucleotides, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV leukemia, and hepatitis. Among fungal diseases treatable by hairpin triplex-forming oligonucleotides are candidiasis, histoplasmosis, cryptococcosis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dermatophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by *Chlamydia trachomatis* or *Lymphogranuloma venereum*. A variety of parasitic diseases can be treated by hairpin triplex-forming oligonucleotides including amebiasis, Chagas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and *Pneumocystis carinii* pneumonia. Also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by hairpin triplex-forming oligonucleotides regardless of whether it is caused by *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*. The infectious diseases identified above can all be treated by hairpin triplex-forming oligonucleotides because the infectious agents for these diseases are known and thus hairpin triplex-forming oligonucleotides according to the invention can be prepared, having a target-forming region that has a nucleotide sequence that hybridizes to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene.

Because DNA can breathe (see, e.g., Ussery and Sinden, *Biochemistry* 32, 6206 (1993)), hairpin triplex-forming oligonucleotides can form triplexes with DNA. See, e.g., Hellene and Toulmi, *Biochimica et Biophysica Acta* 1049, 99 (1990) at page 100.

Cancer cells have a much lower pH than normal cells. See, e.g., Lutz F.

Tietze in *Molecular Aspects of Chemotherapy: Proceedings of the Second International Symposium on Molecular Aspects of Chemotherapy*, Chapter 5 (E.

Borowski and D. Shugar, Eds., Pergamon Press, 1990). Accordingly, one can design hairpin triplex-forming oligonucleotides that are capable of forming hairpin triplexes in cancer cells preferentially. Oligonucleotide activity in cancer cells can be obtained by designing hairpin triplex-forming oligonucleotides to contain more cytosines than thymine, because cytosine must be protonated, preferably at low pH, to form Hoogsteen bonds. It is this difference that provides the opportunity to design hairpin triplex-forming oligonucleotides that preferentially form hairpin triplexes in cancer cells only. For normal cells; methylcytosines, which can be protonated near physiological pH (6.5-6.8), can be used.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended, nor should they be construed, to limit the invention in any manner.

EXAMPLES

Example 1

Synthesis of 5'-S' and 3'-3' Oligonucleotides

The 5'-5' attached oligonucleotides were synthesized on normal solid support (CPG) on which the new oligonucleotide grows in the 3' - 5' direction using normal 5' DMTr 3'-cyanoethylphosphoramidites (Millipore, Burlington, MA). At the 5'-5' attachment site, 3'-phosphoramidites were replaced with 3' DMTr-5'-cyanoethylphosphoramidites (Glen Research, Sterling, VA) and synthesis was continued to obtain the desired oligonucleotide.

The 3'-3' attached oligonucleotide synthesis was carried out on 5' attached solid support (Glen Research) using 3'-DMTr-5'-ss-cyanoethylphosphoramidites. From the 3'-3' attachment site, synthesis was continued with normal monomer synthons. van de Sande et al., Science 241, 551 (1988).

Example 2

Thermal Denaturation Studies

Each antisense oligonucleotide (1.2 μ M) was mixed with equal amount of target in a buffer of 100 mM sodium acetate, pH 5.0, containing 10 mM magnesium chloride, heated to 95°C for 10 min, allowed to come to room temperature slowly and then left at 4°C overnight. The melting temperature of the each sample was measured on a Perkin-Elmer Lambda 2 spectrophotometer attached to a thermal controller. Melting curves were recorded by measuring absorbance at 260 nm as a function of temperature at a heating rate of 0.5 $^{\circ}$ C/min. Melting transition mid-points were determined by plotting dA/dT v.

T. Each value is an average of two independent experiments. Uncertainties in the T° C values is about 1.0 $^{\circ}$ C. The results are displayed in Figure 5 and Table 2. In the RNA sequence, U replaces T. The numbers in parentheses are ΔT_m values relative to the Watson-Crick aps duplex of oligonucleotide SEQ ID NO 2. Triple helices of oligonucleotide SEQ ID NOs 6 and 7 with RNA purine target are not stable and, therefore, are not shown.

TABLE 2
Oligonucleotide Sequences and Thermal Melting Data T_m (°C)

pH 5.0 pH 7.6
SEQ ID NO.

DNA	RNA	DNA	RNA
1+5	41.0	---	45.3
2+5	48.6	32.7	52.0 32.1
3	47.8	---	48.6
4	45.3	---	46.9 --
3+2	63.1	53.3	54.6 41.4
	(14.5)	(20.6)	(2.6) (9.3)
4+2	63.4	53.7	53.3 43.1
	(14.8)	(21.0)	(1.3) (11.0)
6+5	62.5	---	54.2
	(13.9)	(2.2)	(2.2)
7+5	63.3	---	54.8
	(14.7)	(2.8)	

In these UV thermal melting studies, oligonucleotides SEQ ID NOs 3 and 4 showed T_m s of 47.8°C and 45.3°C, respectively, due to the formation of ps duplexes. CD studies confirmed formation of Hoogsteen hydrogen bonding ps hairpin duplexes (Liu et al. supra) below pH 6.0 by SEQ ID NOs 3 and 4. Native polyacrylamide gel electrophoresis experiments revealed the difference between ps and aps (anti-parallel strand) hairpin duplexes (Liu et al. supra). The linear ps and aps duplexes formed by control

oligonucleotides SEQ ID NOs 1 and 2 with the complementary strand have T_s of 41.0 and 48.6°C, respectively (Table 2).

In the presence of the DNA target strand SEQ ID NO 2, oligonucleotides SEQ ID NOs 3 and 4 formed stable triplexes with considerably higher stability than the ps or aps duplexes. The resulting triplexes showed single, cooperative melting transitions with T_ms of 63.1 and 63.4 °C (pH 5.0) for SEQ ID NOs 3 and 4, respectively. Both oligonucleotides formed complexes with 1:1 stoichiometry as determined by electrophoretic mobility shift assay on native polyacrylamide gels, similar to the triplexes formed with control oligonucleotides SEQ ID NOs 6 and 7 Kandimalla and Agrawal, Gene, supra. The triplexes of SEQ ID NOs 6 and 7 with their single-stranded DNA-purine target SEQ ID NO 2 showed T_s of 62.5 and 63.3 °C, respectively. Oligonucleotides SEQ ID NOs 3 and 4 formed triplexes at pH 7.6 in the presence of spermine (Moser and Dervan, Science 238:645 (1987) and Harvey et al. Antisense Res. Dev. 1:307 (1991)), although the triplexes are 10°C less stable than those formed at pH 5.0 (Povsic et al. J.

Am. Chem. Soc. 111:3059-3061(1989); Xodo et al. Nucleic Acids Res. 19:56255631 (1991)) Oligonucleotides containing cytosines require acidic pH conditions for triplex formation. Triplex formation at physiological pH is achieved in the presence of 1 mM spermine. The triplex formed at neutral pH in the presence of spermine is less stable, however, than the triplex formed at acidic pH conditions. Alternately, 5-methylcytosines can be used in order to promote triplex formation at neutral pH without using spermine. Similar results were obtained with oligonucleotides SEQ ID NOs 6 and 7 at pH 7.6. Comparison of the melting profiles and CD spectra of the triplexes SEQ ID NOs 3 and 4 to those of SEQ ID NOs 6 and 7 revealed similar characteristics, suggesting the formation of highly stable triplexes in both the cases (Kandimalla and Agrawal, Gene 149:115-121, Prakash and Kool, J. Amer. Chem. Soc. 114:3523-3528(1992).

Oligonucleotides SEQ ID NOs 3 and 4 exhibited strong binding to the single-stranded RNA target SEQ ID NO 2 (with T_ms of 53.30°C and 53.7°C at pH 5.0. Under the same experimental conditions, the heteroduplex of RNA SEQ ID NO 2 and its complementary strand showed a T_m of 32.7°C (Roberts and Crothers, Science 258:1463-1466(1992). -Triple helices of SEQ ID NOs 3 and 4 with RNA target sequence have 20°C (AT_m) higher thermal stability over the Watson-Crick heteroduplex. The T_m calculated for RNA triplex is about 6 °C higher than AT_m, calculated for DNA triplex (Table 2). This difference in AT_m is about 10 °C higher for RNA triplex at pH 7.6 than at pH 5.0 (Table 2) suggesting a more stable triplex formation with RNA target at physiological pH.

Recent studies suggest that triple helix types of oligonucleotides SEQ ID NOs 6 and 7 with RNA purine target strand are not stable (Kandimalla and Agrawal, Gene 149:115-121(1994). Kandimalla and Agrawal Nucleic Acids Res. 23:1068- 1074 (1995), Robens and Crothers, Science 258:1463-1466 (1992), Han and Dervan, Proc. Natl. Acad. Sci. USA, 90: 3806-3810 (1993), Escude et al., Nucleic Acids Res. 22:5547-5553 (1993), Wang and Kool, Nucleic Acids Res.

22:2326-2333 (1994)). Further studies of the formation of triple helices under different temperature and pH conditions revealed stable triplex formation even when the ps hairpin SEQ ID NOs 3 and 4 and target DNA or RNA strand SEQ ID NO 2 were incubated at 4°C confirming that the triplex forms readily and does not require conformational changes.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Agrawal, Sudhir

(ii) TITLE OF INVENTION: Pyrimidine-Targeting Hairpin
Triplex-Forming Oligonucleotides

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
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(C) CLASSIFICATION:
(viii) ATTORNEY/AGENT INFORMATION:
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(A) TELEPHONE: (312)715-1000
(B) TELEFAX: (312)715-1234 (2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
TCCTTCTTTC TTTTTC 16
(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2: TTTTTCCTT CTCCT 16 (2)
INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: YES
(ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 17..37
(D) OTHER INFORMATION: /note= "nucleotides
17-37 are listed in the 3' -> 5' direction".
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGAAGAAAG AAAAAAGGAG GTTTTTTCTT TCTTCCT 37

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..16

(D) OTHER INFORMATION: /note= "nucleotides
1-16 are listed in the 3'-> 5' direction"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4: AAAAAAGAAA GAAGGAGGAG
GTCCTTCTTT CTTTTT 37

(2) INFORMATION FOR SEQ ID NO:5: (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGAAGAAAG AAAAAA 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCCTTCTTC TTTTTTCTCT CTTTTTCTT TCTTCCT 37

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: TTTTTTCTTT CTCCTCTCT

CTCCTTCTTT CTTTTT 37

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